A STUDY OF MOUSE ENCEPHALOMYELITIS

A Thesis

Presented to

the Faculty of the School of Medicine

Yale University

in Candidacy for the Degree of

Doctor of Medicine

Section of Preventive Medicine

by

Richard Steele Buker, Jr.

1948

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INTRODUCTION

In 1937 Dr. Max Theiler (1) published the first description of a spontaneously occurring paralytic disease of mice and showed that the causative agent was a virus. Theiler and Gard (2) later published a more detailed account of their studies on mouse encephalomyelitis virus. Since Theiler (1) first drew attention to this disease, a number of writers (3,4, 5,6,7,8) have presented evidence indicating that spontaneous paralysis of mice is a geographically widespread, although uncommonly encountered disease. Olitsky (4) has further shown that the causitive agent of this disease, which he named TO virus, is latent in the intestinal tract of most apparently normal mice. Theiler and Gard (2) in 1940 described two separate, distinct clinical and etiological entities of mouse encephalomyelitis. This paper will be concerned with a study of a disease in many respects resembling the myelitic type of disease caused by Theiler's Original virus, which will henceforth be referred to as TO virus.

On November 14, 1947, a paralyzed mouse was noted in a group of animals which had been inoculated intranasally on the previous day with a suspension of mouse lungs being tested for the presence of pneumonia virus of mice (PVM). This animal was sacrificed; its brain and spinal cord, the lungs, and the intestinal contents

with representative portions of jejunum, ilium, caecum, and colon were harvested. Each sample was handled with aseptic technique and subjected to the following procedures:

1. The brain and spinal cord were ground in a mortar, and beef broth added to make a 10% suspension. This was inoculated on the following day into six mice, each animal receiving 0.03 ml. of the broth suspension of nervous tissue by the intracerebral route. Cultures in broth and on a blood agar plate showed no growth in 48 hours. All of the animals which received this material eventually contracted paralytic disease.

2. The lungs were ground, and made into a 10% suspension in broth. The broth and blood agar cultures of this material showed slight bacterial growth. It was inoculated into six mice by the intranasal route, each animal receiving 0.05 ml. of the 10% lung suspension. None of these animals showed any manifestations of disease. Sixty days later, the observation of these mice was terminated. The autopsies were completed negative.

3. The intestinal contents, together with representative samples of different portions of the gut which had been collected, were thoroughly ground, broth was added to make a 10% suspension, and in order to render the material free of bacteria it was centrifuged at 12,000 RPM in the multi-speed attachment of the refrigerated International

machine. Cultures of the supernate showed no growth after 48 hours of incubation. 0.03 ml. of this supernate was inoculated intracerebrally into each of six mice. Two animals displayed paralysis on the 19th day; on the 27th day one animal showed questionable signs of disease. This creature was sacrificed; a 10% suspension of his CNS was passed intracerebrally to six other animals. None of the six showed any signs of disease.

CLINICAL CHARACTERISTICS

Mice which developed paralytic disease following intracerebral inoculation of infectious material showed a definite progression of symptoms. The first harbinger of paralysis was a ruffled appearance of the animal's fur, and at the same time a generalized lassitude on the part of the mouse. These animals usually seemed to be less strong, and less inclined to exert themselves than normal mice. They definitely could not run as fast as healthy mice of a similar age and sex. On the day following the appearance of these generalized symptoms, weakness of one or more limbs usually appeared. This weakness was frequently manifested first by partial paralysis of the extensor muscles of the hind limb, and was soon followed---usually in ten to twenty hours---by flaccid paralysis of the member. The paralysis then spread to involve the fore limbs on the third day following

onset of symptoms.

Paralysis usually became maximal on the fourth or fifth day. Complete quadriplegia was not uncommon, and paralysis of the tail was noted in a few animals. Facial paralysis was noted in several mice, with paralysis of the tongue, and loss of control of the eyeball also noted. (Inability to retract the eyeball and to wink gave rise to severe secondary ophthalmic difficulties in some animals.) In due time, paralyzed limbs underwent considerable atrophy of disuse, and the unused joints became anklyosed in bizarre positions. It should be emphasized that at any point in the progression of symptoms described above, a mouse might cease to get worse, and the paralysis regress slightly, or it might persist as it was at the height of the disease in that particular animal.

An analysis to the first 136 mice which became paralyzed as a result of intracerebral inoculation was made in order to determine the most frequent site of initial paralysis. Of the 136 mice, 129 first presented paralysis of one or both hind limbs. The remaining seven first manifested disability with fore limb paralysis. It is of interest to note that of those mice with initial paralysis in only one hind leg, the right leg was involved twice as frequently as was the left limb. All mice were inoculated in the left cerebral hemisphere.

FIGURE 1.

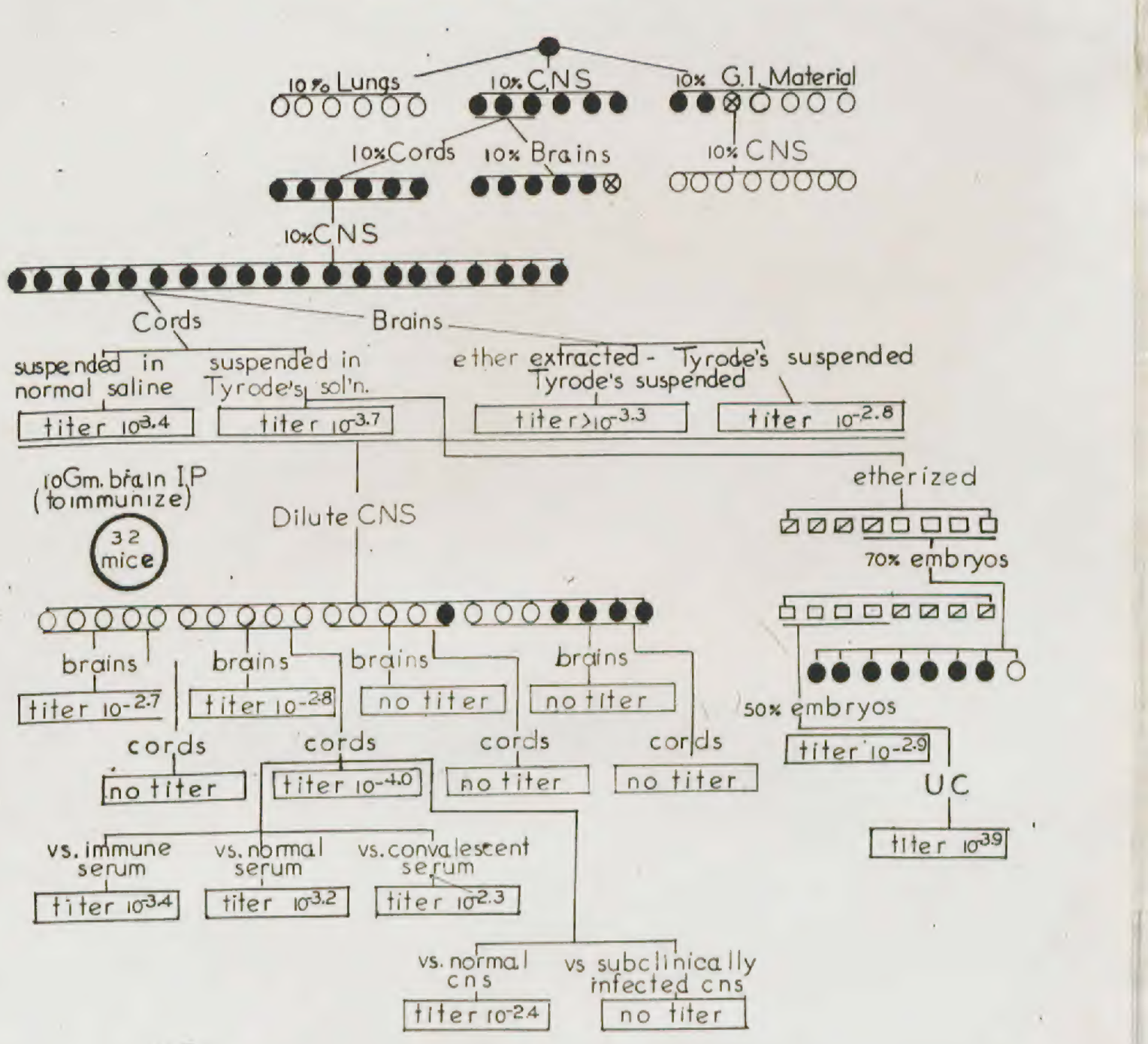

Fig. 1. A diagramatic synopsis of virus passages carried out in this study.

Figure 2. represents graphically all the mice becoming paralyzed during the course of this study. No instance of encephalomyelitis was encountered before the ninth day following intracerebral inoculation, and by the twenty-first day following inoculation, 93% of all the paralysis had occurred. Observations in all instances were continued for a period of at least thirty days. Three animals were observed to develop paralysis after the thirtieth day following inoculation.

Theiler (1) in describing his original disease stated that the resulting paralysis of mice suffering from the myelitic disease in "most instances leads to death". On the basis of observations in this present study it is believed that most deaths are due not to the infection itself but chiefly to dehydration and inanition secondary to severe paralysis which prevents the mice from reaching food and water. The fact that less than 10% of mice appear to have succumbed to the disease per se is in support of this view. If an animal was observed to get progressively dehydrated, with no evidence of cyanosis or respiratory distress, its death was regarded as due to secondary causes.

As these experiments were done exclusively in male mice, there was an inordinate amount of fighting among them from the time they were six to eight weeks old.

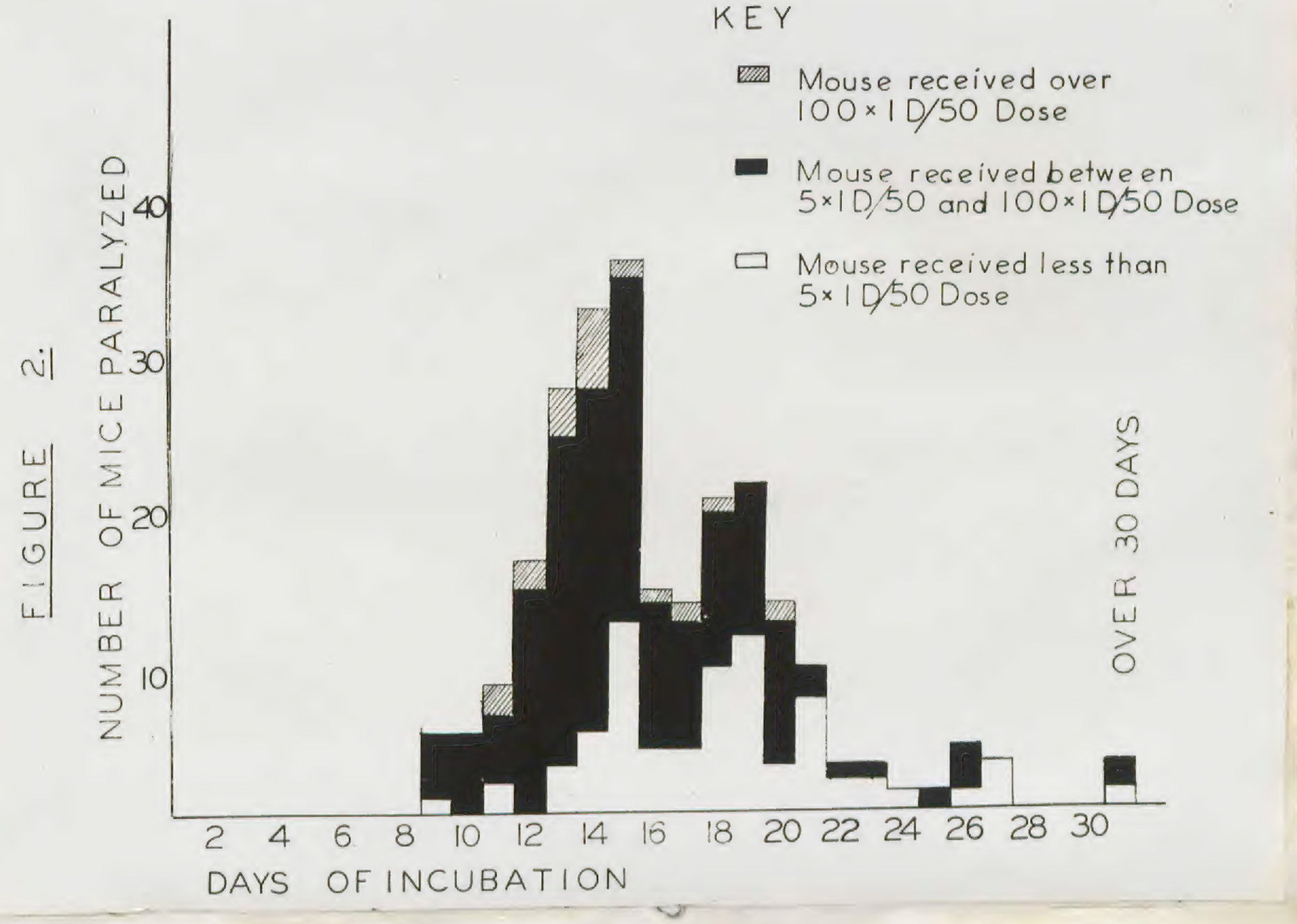

Fig. 2. Length of incubation period.

Frequently paralyzed mice fell victims to belligerent cage mates and died of obvious injuries. Despite secondary causes, the writer feels that 75% of all paralyzed mice will live if given reasonable nursing care.

Gard (9) showed that the mean length of incubation time could be relied upon, when dealing with the type of mouse encephalomyelitis caused by FA virus, to give a reasonable estimate of the infectivity titer of the dose given the animals. Graphic illustration of the findings in this study with respect to the incubation period are portrayed in Figure 2. The open squares represent the mice coming down with the disease that received less than 5 times the ID/50 dose. The black squares show the mice that got more than 5 and less than 100 ID/50 doses of virus. The cross-hatched squares indicate mice receiving over 100 ID/50 doses. As can be seen from the graph, the amount of virus given to an animal seemed to have some bearing on the length of incubation period in this disease. However, evaluation of the present data indicates that the scatter is so great that for all practical purposes the average incubation period in anything less than a very large number of mice is not significant. The various procedures used to purify virus which will be described later in this paper also do not appear to effect the incubation period of this disease.

METHODS OF PREPARING AND PURIFYING THE VIRUS

In order to ascertain an efficient method of suspending virus, the following experiment was done: a weighed batch of infected brains was put in a mortar and thoroughly ground without adding any abrasive or diluent. When the nervous tissue had been reduced to a homogenous paste, approximately half of the material was transferred to a weighed mortar. The exact weights of each aliquot were then determined and recorded as a basis for subsequent calculations.

To the aliquot of brain in mortar #1 was added about 20 volumes of ether. The ether was thoroughly worked into the brain tissue with the pestle, in the hope of achieving a maximal extraction of lipid substance. In this and subsequent work it was found that in order to obtain maximal ether extraction, a sample should be worked a half hour (this length of time for a 10 Gm. sample, and longer with larger masses of tissue). During the extracting procedure, the brain paste undergoes a definate change in its physical character. Before the ether is added the material is ordinarily of a pink color, thick, viscous, and quite adherent to the mortar and pestle; as the material is extracted with ether it becomes much less adherent to the mortar, looses its fluidity, and becomes quite friable, resembling putty or dry dough rather than a thick fluid. Its color also tends to deepen to

a dark pink or light brown. It is of value to observe the change of physical characteristics to estimate progress toward maximal extraction.

The brain sample in mortar #1 was extracted twice with ether. The ether was pipetted off, the two samples combined and centrifuged at 1500 RPM for 10 minutes. This produced a water-clear, supernatant fluid, and a small white pellicle of sediment. The sediment was returned to the extracted brain material, and the ether allowed to evaporate off in weighed petri dishes. This and other similar experiments have lead us to expect a residue after evaporation approximating 25% of the weight of the extracted brains. When spinal cords were similarly extracted the residue left following evaporation of ether was about 50% of the total weight of the raw material.

Following double ether extraction the brain material was suspended in enough Tyrode's solution* to make a 10% suspension in terms of the original unextracted brain weight. Ether was then shaken into this aqueous suspension and the mixture left overnight in the refrigerator. On

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* The Tyrode's solution used in the experiments described in this paper was made up in two concentrated stock solutions, one solution containing the carbonate and phosphate anions, the other containing the remaining salts. Both were Seitz filtered, and stored in sterile flasks, aliquots being diluted as needed. The formula used was that given in Best and Taylor's: Physiological Basis of Therapeutics, third edition, p. 265.

the following day the ether layer was pipetted off, and on evaporation this third ether extract showed no perceptable residue. The aqueous suspension was cleared by low speed centrifugation and the supernate was diluted serially to 10^{-1}, 10^{-2}, 10^{-3}. Samples of each of these dilutions were tested in seven mice, each mouse receiving 0.03 ml. of inoculum intracerebrally. The serial dilutions were made directly before inoculating the mice.

The brain sample in mortar #2 was also made into a 10% suspension with Tyrode's solution. It was interesting to note the marked difference in turbidity between this sample of material and the ether extracted aliquot; one might roughly compare the ether extracted 10% material to the 1% straight dilution as being in the same realm of turbidity. This material was inoculated, in the same test dilutions as sample #1, into mice of the same age and sex as received the ether extracted material. Sample #2 suffered the same temperature and time ranges as the ether extracted sample #1, but was not treated with ether at any point.

In this laboratory infectious material is usually suspended in physiological saline prepared in the Culture Media section of the Department of Bacteriology and Immunology. Tyrode's solution, however, seems

definitely to have a less rigorous effect on mice who receive it intracerebrally than saline. When inoculating large numbers of animals intracerebrally with a saline vehicle, it is not unknown to have a 10% mortality within a few hours of inoculation, yet the same size dose of Tyrode's suspended agent will frequently result in less than a 2% immediate mortality as a result of the trauma of inoculation. Therefore, an experiment was planned to determine if Tyrode's solution would be measurably different from saline as a vehicle for this virus.

For this experiment spinal cords were used from the same infected mice from which the brain material was taken in the preceding experiment. The cords were weighed and made into a paste in the same manner as the brains. The material was divided into two aliquots, and the weight of each determined; one was suspended in Tyrode's solution, the other in the standard normal saline. Serial dilutions were made just before inoculation; 10^{-2}, 10^{-3}, and 10^{-4} dilutions were tested in the same manner as described for the brain material. The saline suspension was carried through dilutions in saline, and the Tyrode's suspended material was serially diluted in Tyrode's solution. These experiments on the cord material were run concurrently with those on the brain material. In this way it could be shown at one time the relationship between various methods of preparation and the difference between cords and brains as a source of virus.

The mice in these titrations were observed for thirty days, and the ID/50 titers (16) were calculated at the end of that time. The results were as follows.

TABLE I

MODE OF PREPARATION AND MATERIAL TESTED	CALCULATED TITERS
Brain extracted with ether and suspended in Tyrode's solution	$>10^{-3.3}$
Brain suspended in Tyrode's soln.	$10^{-2.8}$
Cord suspended in Tyrode's soln.	$10^{-3.7}$
Cord suspended in Saline soln.	$10^{-3.4}$

The results shown in Table I strongly suggest that extracting with ether before adding diluent has a salutary effect on infectivity titer. The effect of ether extractions on the physical properties of a suspension of central nervous tissue has been described elsewhere in this section.

Infected nervous tissue suspended in Tyrode's solution was found to have a 0.3 log. higher infectivity titer than its saline suspended control.

Spinal cord tested under similar conditions as brain material shows eight times (0.9 log.) as high a concentration of infectious agent. (This spinal cord material was taken from the same animals as the brain material.)
In the light of these findings ether extraction, followed by Tyrode's solution as a diluent was generally employed in in the routine preparation of virus.

GROWTH OF VIRUS

Some interesting phenomena have been observed in work done with other viruses as regards their multiplication in the host. Particularly influential to the writer was Curnen and Horsfall's (10) work with PVM, in which they showed that the highest titer of virus was obtained chronologically some time before the maximal effect of the disease was manifested clinically in mice. An experiment was planned to sample the titer of virus at five day intervals following inoculation of a group of mice. Twenty-four healthy seven-week-old male white mice obtained from Tumblebrook Farms, and observed daily for four weeks prior to inoculation were used. Each animal received 0.03 ml. of a 10% suspension of CNS harvested from paralyzed mice of the fourth virus passage. (See Figure 1.)

Five animals were selected at random five days after inoculation, these were sacrificed, the brains and cords harvested separately in a sterile manner and the material prepared immediately by grinding and ether extraction. It was then suspended in Tyrode's solution and allowed to stand overnight under ether. The Tyrode's suspension of the material was titered in mice---as described above. The brains and cords were suspended and titered separately. On the tenth day after inoculation five more animals were

TABLE II

MATERIAL AND TITERS	DILUTIONS TESTED 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
FIVE DAY POOL *					
Fate of mice receiving brain Titer $10^{-2.7}$	4/6**	7/7	2/7		
Fate of mice receiving cord Titer less than $10^{-2.}$		0/7	0/7	0/7	
TEN DAY POOL					
Fate of mice receiving brain Titer $10^{-2.8}$	3/5	5/7	4/7		
Fate of mice receiving cord Titer $10^{-4.0}$		5/7	5/7	5/6	
FIFTEEN DAY POOL					
Fate of mice receiving brain No titer calculated	0/7	1/7	0/7		
Fate of mice receiving cord No titer calculated		0/7	2/7	0/7	
TWENTY DAY POOL					
Fate of mice receiving brain Titer less than $10^{-2.}$	2/7	0/7	0/7		
Fate of mice receiving cord Titer less than $10^{-3.}$		0/7	0/7	0/7	

* The five and ten day material was tested in mice five to six weeks old; the fifteen and twenty day material was tested in mice three to four weeks old.

** The numerator is the number of animals showing definite paralysis; the denominator represents the number of mice which were used in testing a given dilution. Those cases in which the denominator is less than seven can be explained by mice dying which were not seen to definitely have paralysis, or mice escaping; in both events the test was calculated as though these animals had never been used.

selected at random and treated exactly as those mice which were harvested five days following inoculation. Thirteen days after inoculation of the remaining thirteen animals, one mouse came down with paralysis and two came down on the fourteenth day; on the fifteenth day, one of these three paralyzed mice was included arbitrarily with four selected at random from the asymptomatic mouse pool to represent the level of virus activity after fifteen days of incubation. During the next five days three of the six remaining asymptomatic mice (one mouse having died as a result of the trauma of inoculation) developed paralysis, and one animal died of the disease. On the twentieth day the surviving seven mice were pooled and harvested---four of the seven being paralyzed. They were treated in the same manner as the preceding three batches of mice. Each titration was followed for thirty days, at which time the virus titers were computed. The results were of such interest as to warrant complete reports of the number of paralyzed animals, as well as the final calculated titers.

An analysis of Table II revealed two observations of interest. The first, an expected result, was that virus in high titer would be found in asymptomatic mice late in the incubation period of the disease. (See Figure 3.) This was quite in keeping with the results that Bodian and Cumberland (11) have reported in a study of the growth

FIGURE 3

Comparison of Virus Titer and Onset of Paralytic Disease in a Pool of 24 Mice

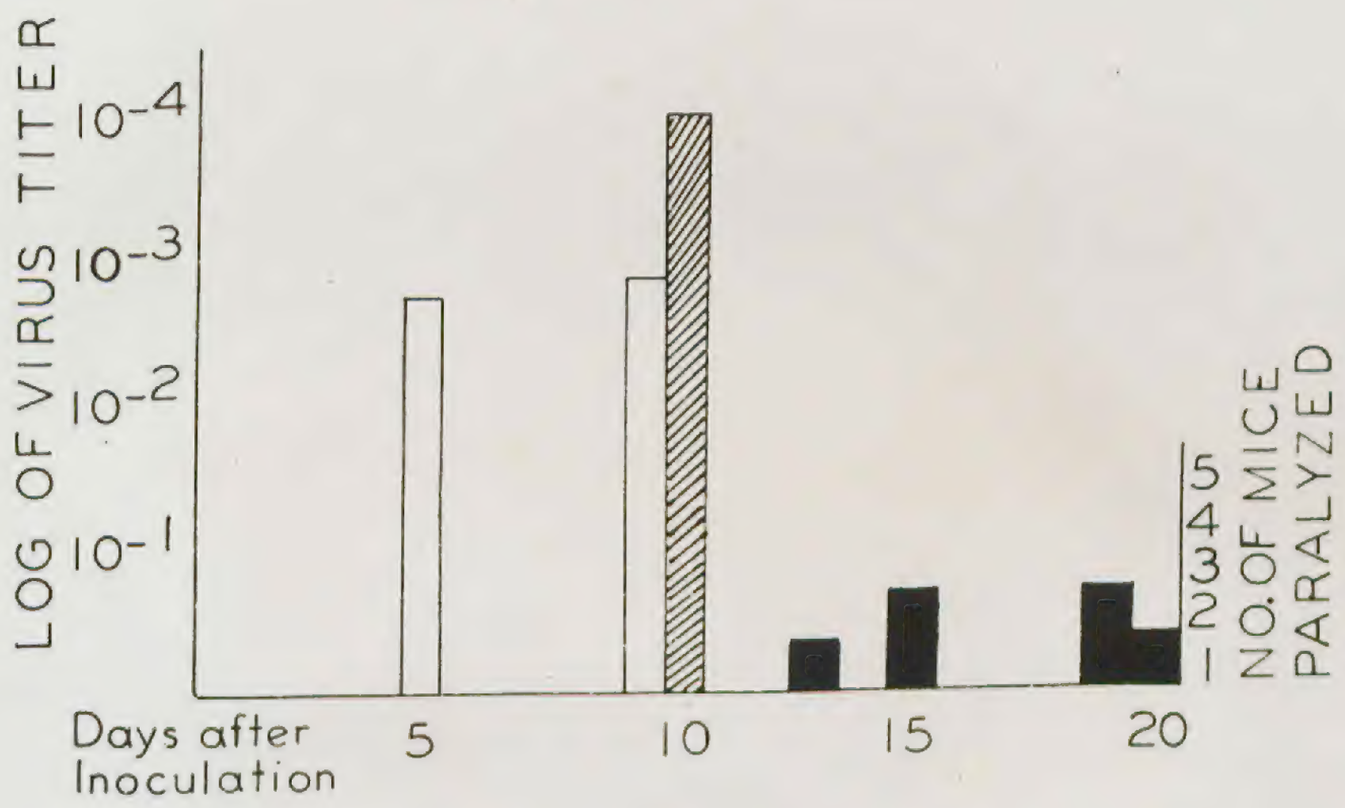

Fig. 3. Virus titers determined at five day intervals in a pool of twenty-four mice.

characteristics of Lansing virus in the monkey's central nervous system. The second result was the paradoxical appearance of virus activity at considerable dilution, when none could be demonstrated in more concentrated preparations. This resembles similar observations by Taylor (15) with influenza virus. He interpreted his findings as evidence of a serum antibody present in sufficient concentration in the lower dilutions to block entirely the action of the virus, but in greater dilutions, the antibody having been diluted beyond an effective concentration, the virus is left free to exert its effect. Elsewhere in this paper evidence will be presented to show that circulating antibody was not clearly demonstrable in the disease under discussion. It seemed reasonable to postulate, however, the possibility that some sort of fixed tissue antibody was present in the central nervous systems which had apparently developed resistance to the virus. An experiment was designed to investigate this hypothesis.

ANTIBODY

Theiler and Gard (2) reported that they could find only suggestive evidence for the presence of humoral antibodies against TO virus. This paper will present additional similar evidence to suggest the same conclusion.

A search for antibody was carried out in both serum and central nervous system tissue. In the tests for antibody reported herein a single suspension of 10% spinal cord titering $10^{-4.0}$ was used. Material being tested for antibody was kept constant, and varying amounts of antigen were used.

Humoral Antibody:

An attempt to immunize mice was carried out in the following manner. Forty mice were bled, on two occasions a week apart. Following the second bleeding the 32 surviving animals were inoculated intraperitoneally, with a 40% suspension of infected mouse brain. Each animal received a 0.4 ml. dose. At six day intervals similar doses of the 40% brain were inoculated. Three doses were given in all, and it is estimated that each animal received a minimum of 1000 times the ID/50 intracerebral dose. Thirteen days following the last dose of virus the mice were bled again. At no time did any animal show signs of paralytic disease; however, it was interesting to observe the profound change in behavior that developed in these mice. They persisted in fighting continually, whereas an equal sized group of unimmunized mice of similar age, sex, number and origin in the adjacent cage did only the usual amount of fighting. In fact, during the eight weeks time in which these mice were observed, the immunized mice killed 75% of their

number by fighting alone.

Blood was obtained from these mice by cardiac puncture, and the blood drawn and deposited under oil--- a procedure which seems to minimize frothing and subsequent hemolysis. The sera were kept frozen in the dry icebox.

Another group of paralyzed mice were bled ten to twenty days following the onset of illness; these animals had no preinoculation control serum collected.

Neutralization tests were carried out in the following manner. An ether extracted, Tyrode's suspended, frozen and thawed suspension of infected mouse cord which titered $10^{-4.0}$ was used as a virus in the neutralization tests. First the three unheated samples of serum (1. the preinoculation bleeding; 2. the I.P. immunized mouse serum; and 3. the convalescent mouse serum) were diluted with Tyrode's solution to give a 1:4 serum concentration. Three sets of three tubes each were arranged, the first tubes receiving 0.5 ml. of the dilute sera. The remaining tubes received 1.8 cc. each of the sera. To the first tube of each series was added an equal volume of the 10% virus suspension, and from the virus and serum mixture serial transfers of 0.2 ml. through the other two tubes were carried out. This gave virus diluted 1/20, 1/200, and 1/2000 in serum diluted 1:4. The virus and serum

mixtures were incubated at 37° C. for two hours and then immediately inoculated into the mice. On the 30th day after inoculation titers were calculated. (See Table III)

As can be seen from the results recorded in Table III there is an apparent inhibition of virus activity by the convalescent serum. However, in evaluating this finding it should be understood that this serum was inadvertently contaminated, (a fact learned after the test was underway). Over half the animals had signs of meningitis and seven mice died of meningitis. All signs of infection had subsided by the seventh day and the test was allowed to continue. The apparent inhibition of virus activity by the convalescent serum could conceivably be explained on the basis of the bacterial contamination.

Fixed Antibody in the Central Nervous System:

In the experiment ascertaining the effect of time on the growth of the virus some suggestive evidence of a fixed inhibiting agent in the brain was demonstrated. (See Table II) It was therefore felt expedient to investigate the virus inhibiting properties of central nervous tissue. In order to reproduce as nearly as possible the conditions observed in testing the virus which had been incubated in mice for fifteen days, the following procedures were carried out.

As controls, five apparently normal, five to six weeks old male, white Swiss mice (following observation for two weeks for signs of illness) were selected from a pool of animals and killed by exsanguination under light ether anesthesia. Their brains and cords were harvested in a sterile manner, ground without etherizing and made into a 10% suspension with Tyrode's solution. 1.8 cc. of the 10% suspension was put into each of three sugar tubes. 0.2 cc. of the same infectious cord used for the serum neutralization tests, was pipetted into the first tube and thoroughly mixed. Serial dilutions, (changing pipettes with each passage) of 0.2 ml. were made through the other two tubes. The material was incubated at 37°C. for one hour, and then inoculated into mice, seven animals being used to test each dilution, and each animal receiving 0.03 ml. of test mixture intracerebrally.

A similar preparation of the brains and cords of approximately ten mice who were believed to have had inapparent infection of the central nervous system* was prepared in essentially the same manner as were the normal controls. The only difference was that the "sub-clinically infected" central nervous tissue was taken

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* Mice which had survived over thirty days with no sign of disease after receiving intracerebral doses of virus known to contain over five times the calculated ID/50 dose.

from the dry icebox where it had been frozen for several weeks, whereas the normal mouse material was prepared from the fresh, unfrozen state. The same virus dilutions as were used in the control samples were employed, and the test was run simultaneously with its control. The results of this test are to be found in Table III.

TABLE III

MATERIAL TESTED	CALCULATED TITER
Cord	$10^{-4.0}$
Cord vs. Normal Serum	$10^{-3.4}$
Cord vs. Immune Serum	$10^{-3.2}$
Cord vs. Convalescent Serum	$10^{-2.3}$
Cord vs. Normal Central Nervous System	$10^{-2.4}$
Cord vs. Subclinically Infected Central Nervous System	$10^{-1.7}$ or less

It can be seen from the results in Table III that mixing virus with 1:4 serum and incubating for two hours at 37° C. causes some diminution of infectivity. Serum from preimmunization bleedings compared with immune serum for anti-virus activity shows a difference believed to be insignificant in this method of testing. The results obtained with convalescent serum are interesting, and are tendered as suggestive evidence for the presence

of antibody. The results shown in Table III demonstrate a rather marked inhibition of virus by a 10% suspension of what was believed to be normal central nervous system tissue. The "subclinically infected" CNS versus virus titration demonstrated only one case of mouse paralysis in the 10^{-2} dilution and a hypothetical titer of $10^{-1.7}$ or less is reported. It seems reasonable to postulate that the anti-virus property of this material was greater than that of any other material tested.

CONCENTRATION OF VIRUS

Concentrated virus from the central nervous systems of mice is exceedingly tedious and expensive to prepare. It was thought worthwhile, therefore, to investigate the possibilities of concentrating virus from infected chick embryo material.

Riordan and SaFleitas (12) reported that TO virus could be grown in developing chick embryos. They found that an ID/50 titer of $10^{-3.0}$ could be obtained in the embryos of eggs incubated for twelve days after inoculation with virus. Riordan's (13) suggestion, that eggs incubated six to ten days be inoculated directly into the allantoic cavity and the virus be allowed to grow for ten or more days before harvest, was followed in this experiment. A suspension of infected mouse CNS

known to have a titer of $10^{-2.6}$ was used as the initial inoculum. Eight ten day chick embryos were each inoculated with 0.05 ml. of the suspension material which was shown by blood agar and broth cultures to be bacteria free. On the tenth day following inoculation four of the embryos were alive and active; these were sacrificed and the whole embryos harvested. The embryos were treated by maceration in the Waring blendor and the addition of 1/3 their volume of Tyrode's solution. The mash was extracted with ether. The watery extract was drawn off and inoculated into mice, four mice being tested with a 10^{-1} dilution, and four receiving a 10^{-2} dilution. At the same time, eight more six day embryos were inoculated in the same manner as were the first group. In due time all but one mouse came down with typical paralytic disease.

The embryos of the second passage were harvested after the virus had incubated eleven days. These embryos were macerated as before; this time an equal volume of Tyrode's solution was added to them. Ether extraction was carried out, and the aqueous layer after centrifugation at 2500 RPM for 1/2 hour was drawn off. Freezing and thawing of the clear, wine-red watery extract was the next step in the purification. The extract was frozen on dry ice, then thawed in tepid water five times. After the freezing and thawing a cloud of brown precipitate

was seen to have formed. This was removed by centrifugation at 2500 RPM for 20 minutes after the second and fifth freezings. After the fifth freezing, thawing, and centrifugation, the supernate appeared as a slightly opalescent colloidal suspension with a brownish color. The sediments from these low speed centrifugations were discarded.

Following centrifugation of the fifth thawing the supernate described above was tested in mice and found to have an ID/50 titer of $10^{-3.2}$, the titer being calculated in terms of the weight of the original embryo. Two more freezings and thawings with low speed centrifugation were carried out, bringing down an additional amount of the brownish colloidal material. About 120 ml. of this concentrated material being on hand from the four eggs harvested, an aliquot was taken and subjected to a spinning in the ultracentrifuge --- a sedimenting force of 100,000 times gravity being exerted for one hour (600 revolutions per second in the machine that was used)*. A laminated pellicle was noted in each tube, and a clear amber supernatant fluid was discarded. The pellicles (which were small) were homogenized as well as possible with the rubber "squeegee" tipped glass rod, and the sediment suspended in 5.0 ml. of Tyrode's solution calculated to be 1/10th

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* The writer is indebted to Mr. George Roberts for his help in carrying out the ultracentrifugation procedure.

of the volume of the aliquot ultracentrifuged. The sediment was at first readily suspended in 1/3rd of the volume used, but for convenience in calculation and to have adequate material for testing the sediment was diluted to the final volume of 5.0 ml.

After allowing small particles of unhomogenous material to settle out, a supernatant suspension of slightly pearly, opalescent appearance was obtained. This was found to have an ID/50 titer in mice of $10^{-3.9}$. This figure represents the infectivity of the suspension of ultracentrifuged sediment. The titer of the 50% suspension before ultracentrifugation was determined, and found to be $10^{-2.9}$. (This is equivalent to an ID/50 titer of $10^{-3.2}$ on the basis of the original embryo weight.) These findings indicate clearly that the rigorous methods of preparation did not diminish infectivity in a measurable degree.

It was also thought worthwhile to make a survey of the effect of the general procedure described above on nervous tissue. A pool of unassayed, infected mouse brains and cords, weighing 37.5 Gm., and consisting of central nervous systems of over 100 animals was collected. This material was put through double ether-extraction and successive freezings and thawings with several low speed centrifugations. The slightly pinkish-brown, opalescent suspension obtained

following this treatment was ultracentrifuged in the same manner as was the chick embryo. The resulting supernatant fluid appeared to contain all the color. It was only with the most careful scrutiny that the exceedingly small gelatin-like pellicle could be seen. It is believed that this material could have been suspended in less than 1/30th of the volume of the original nervous tissue, but for purposes of standardization and workable volumes it was diluted up to 1/10th the volume; that is, it was made up to a total volume of 3.7 ml. This material was only very slightly opalescent, and quite colorless. A control sample of 12.5 Gm. of normal mouse CNS was similarly treated, and was indistinguishable from the infected material in physical properties.

DISCUSSION

In the first part of this paper reference was made to some of the reports in the literature of observed instances of spontaneously occurring encephalomyelitis in mice. An attempt has been made critically to evaluate some of the properties of a strain of encephalomyelitis isolated at this laboratory. When comparing this virus with that described by Theiler and Gard (2) it seems reasonable to postulate that a new strain closely resembling in many respects the other described strains of TO has been isolated.

The characteristics of this strain in which it differs from others studied elsewhere are its longer minimal incubation period, its less lethal character, and its somewhat higher infectivity titer. Theiler and Gard (2) stated that the TO virus of their myelitic type of disease never showed a titer over 1/000, whereas the strain described in this paper occasionally had titers in the neighborhood of 1/10,000.

Also of interest is Theiler and Gard's (2) finding that virus was demonstrable in the central nervous systems of mice who had inapparent infection up to 163 days after inoculation, whereas it was found here that after fifteen days there is evidence that the central

nervous systems of mice with inapparent infection not only contain no demonstrable virus, but in fact seem to have some anti-virus activity.

The evidence for the presence of an inhibitor of TO virus in the central nervous systems of mice with subclinical infections is not complete, and it is felt to be only suggestive at the present time. It would be interesting to learn what effect ether extraction with freezing and thawing of central nervous tissue has on the inhibiting agent. Also the antibody content of nervous tissue from convalescent mice should be determined, as well as the anti-virus action of the CNS's in old and in very young uninoculated mice.

For some time there has been considerable interest in perfecting simple methods of purifying low titer neurotropic viruses. Herrarte and Frances (14) reported in 1943 that ether could be effectively used as an adjunct in purifying Lansing virus. They gave good evidence to show that ether, a bacteriocidal agent, was at the same time harmless to Lansing virus. In this study ether was used as a bacteriocidal agent, and also utilized to extract lipid substances from virus-bearing tissue. From brain 25% of the weight mass could be extracted, from cord about 50% of the mass, and

chick embryo, though not so extensively studied, seems to give up about 5% of its mass to ether. It is of interest that the infectivity titer appears to be enhanced by ether extraction. Centrifugation combined with freezing and thawing were found to be exceedingly efficatious in clearing thick suspensions without detracting from the virus titer. Finally, ultracentrifugation proved useful in the final step of virus concentration.

Tyrode's solution appeared to be somewhat superior to saline as a vehicle for virus. The Tyrode's solution used in these experiments was in effect an isotonic solution buffered at pH 8.2 (these determinations were done with the Beckman pH meter). Gard (8) has pointed out that FA virus demonstrated maximal stability at about pH 8. Although never proved, it seems reasonable to suspect that TO virus would be more efficaciously suspended in alkaline buffered diluent than in the conventional pH 6.5 to pH 6.8 laboratory saline solution.

SUMMARY

1. A strain of mouse encephalomyelitis virus similar to TO virus is described. It has an average incubation period of nine to twenty-one days, with 7% of the animals comihg down after twenty-one days. The disease is fatal in about 10% of the animals, the remainder having residual paralysis with secondary atrophy of the involved members. The disease is characterized by onset in the hind limbs with progression up the animal to fore limbs and face. The clinical symptoms take about four days to reach their maximum.

2. This virus can be efficiently suspended by extracting the infected tissue with ether and suspending it in Tyrode's solution.

3. A high titer of virus can be extracted from central nervous systems ten days following intracerebral inoculation in the absence of any clinical signs of disease.

4. Clear evidence for humoral antibody was not obtained.

5. There is suggestive evidence presented for the existence of fixed antibody in the brains of subclinically infected animals.

6. This virus can be grown readily in eggs by th e

intra-allantoic route of inoculation. Whole embryos demonstrated an ID/50 titer of $10^{-3.2}$ when assayed in mice by the intracerebral route.

7. Freezing and thawing combined with low speed centrifugation are very efficatious in purifying virus-containing suspensions without diminishing the virus activity of the suspension.

8. Ultracentrifugation in a gravitational field of 100,000 times g. will effectively aid in concentration of virus.

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